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Cell type-specific endometrial transcriptome changes during initial recognition of pregnancy in the mare

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INHALTVERZEICHNIS

Abstract

Previous endometrial gene expression studies during the time of conceptus migration did not provide final conclusions on mechanisms of maternal recognition of pregnancy (MRP) in the mare. This called for a cell type-specific endometrial gene expression analysis in response to embryo signals to improve understanding of gene expression regulation in context of MRP. Laser capture microdissection was used to collect luminal epithelium (LE), glandular epithelium (GE), and stroma from endometrial biopsies from Day 12 of pregnancy and estrous cycle, respectively. RNA-sequencing showed greater expression differences between cell types than between pregnancy and oestrus cycle. Differences between pregnancy and oestrus cycle were mainly found in LE. Comparison with a previous RNA-seq data set for whole biopsy samples revealed a specific origin of gene expression differences. Genes specifically differentially expressed (DE) in one cell type were found, which were not detectable as DE in biopsies. Overall, this study revealed spatial information about endometrial gene expression during the phase of MRP. The conceptus induced genes involved in blood vessel development, specific spatial regulation of the immune system and growth factors, and regulation of prostaglandin synthesis, prostaglandin receptors, specifically PTGFR in the context of prevention of luteolysis.

Equus caballus, laser capture microdissection, maternal recognition of pregnancy, RNA-seq, uterus

Zusammenfassung

Vorherige Studien über die Genexpression während der embryonalen Früherkennung (EFE) haben keine finalen Schlussfolgerungen über den Mechanismus der maternalen Erkennung der Trächtigkeit bei der Stute gebracht. Daher wurde eine Studie mit zellspezifischer Analyse der Genexpression im Endometrium während der embryo-maternalen Kommunikation in der Frühgravidität durchgeführt. Dazu wurden am Tag 12 der Trächtigkeit und des Zyklus Endometriumsbiopsien genommen und mittels Laser Mikrodissektion das luminale und glanduläre Epithel sowie das Stroma voneinander getrennt. Die RNA-Seq zeigte grössere Unterschiede zwischen den verschiedenen Zelltypen als zwischen der Trächtigkeit und dem Zyklus, die vor allem im luminalen Epithel zu sehen waren. Der Vergleich mit den Ergebnissen der RNA-Seq einer vorhergehenden Studie, bei der die Endometriumsbiopsien als Ganzes analysiert wurden, zeigte, dass die Unterschiede vorwiegend zelltypspezifisch sind, d.h. Differenzen wurden nur in bestimmten Zellen gefunden. Zusammenfassend liefert diese Studie Informationen zur zelltypspezifischen Genexpression während der maternalen Erkennung der Gravidität bei der Stute. Der Embryo aktiviert Gene im Zusammenhang mit dem Gefässwachstum, der spezifischen lokalen Regulation des Immunsystems, von Wachstumsfaktoren sowie der Synthese von Prostaglandinen und deren Rezeptoren, insbesondere dem Prostaglandinrezeptor F, zur Hemmung der Luteolyse.

Equus caballus, Laser Mikrodissektion, maternale Erkennung der Trächtigkeit, RNA-Seq, Uterus

Introduction

In many mammals, particularly those with late implantation, maternal recognition of pregnancy (MRP) is a fundamental step for successful gestation. The conceptus must signal its presence to the maternal organism to prevent luteolysis which would lead to embryo loss (Allen 2001b). This mechanism has been unraveled to a large extent in most of the large domestic animal species but not in equids (Allen 2001a; Allen and Wilsher 2009). The pig embryo, like the equine conceptus, secretes considerable amounts of estrogen which has been proven as responsible for MRP in the sow but not in the mare (Wilsher and Allen 2011; Bazer 2013).

The major steroid produced during early pregnancy by the equine conceptus is 17 alpha-hydroxyprogesterone, which also could be involved in prevention of luteolysis and conceptus development (Betteridge 2000). Increased expression of potential receptors for this specific progesterone metabolite has been shown in the endometrium on day 12 of pregnancy (Merkl *et al.* 2010). The same study investigating the effects of estrogen found out that intrauterine administration of plant oils at day 10 after ovulation inhibits luteolysis in mares (Wilsher and Allen 2011). Although the exact mechanism is unclear, it is likely that the lipids contained in the studied plant oils modulate the synthesis or release of prostaglandins from the endometrium (Wilsher and Allen 2011).

The ruminant embryo produces a specific type I interferon, interferon tau (IFNT), which prevents the release of luteolytic prostaglandin $F_{2\alpha}$ (PGF2a) (Roberts 1996). Trophoblast IFNT prevents estrogen-induced increases in oxytocin receptor (*OXTR*) gene expression in the endometrium by repression of estrogen receptor alpha (*ESR1*) expression in the endometrial luminal epithelium (Spencer and Bazer 1996). The equine conceptus does not produce alpha or omega interferon molecules, but delta interferons (Baker *et al.* 1991; Cochet *et al.* 2009). They have been suggested to play a role in embryo-maternal interaction but not in MRP in the mare (Cochet *et al.* 2009). Similar to ruminants, endometrial concentrations of OXTR determine uterine PGF2a secretion in cyclic mares and endometrial OXTR concentrations as well as receptor affinity were reduced in early pregnancy by the presence of the conceptus (Sharp *et al.* 1997; Starbuck *et al.* 1998). However, the pulsatile endometrial release of PGF2a into the peripheral circulation, which is the initial signal for luteolysis (Aurich 2008), is someway suppressed by the presence of the equine conceptus and/or its secretions (Goff *et al.* 1987; Wilsher and Allen 2011).

A particularity that only the equine embryo is showing, is the self-induced mobility throughout the entire uterus between days 6 and 17 after ovulation by peristaltic contractions of the uterus in response to prostaglandins derived from the embryo (McDowell *et al.* 1988; Stout and Allen 2001; Allen and Wilsher 2009). One of the conceptus prostaglandins is PGF2a having a local function in stimulating the myometrial contractility but also the rapid expansion of the early equine blastocysts (Stout and Allen 2001; Stout and Allen 2002). Studies in sheep have shown an important role of prostaglandins in conceptus development and regulation of endometrial function (Dorniak *et al.* 2011; Dorniak *et al.* 2012). After the systemic administration of the synthetic prostaglandin inhibitor, flunixine meglumine, conceptus movement is abolished resulting in luteolysis (Stout *et al.* 2000).

In context of the migration, the equine conceptus has another unique property that is its typical spherical shape given by the glycoprotein capsule (Rivera Del Alamo *et al.* 2008;

Allen and Wilsher 2009) present between days 7-21 of gestation (Betteridge 2000). This capsule makes the conceptus to resist the myometrial forces that move it through the uterus (Stout *et al.* 2005). McDowell et al. first demonstrated the importance of embryo mobility in conjunction with MRP by ligation of one uterine horn or one uterine horn and the uterine body which led to return to estrus (McDowell *et al.* 1988). Later on, Rivera Del Alamo et al. demonstrated that the introduction of an intrauterine device (IUD, water-filled plastic ball, diameter 20 mm) induced a prolonged luteal phase in 75% of the mares. The mechanism of action is still unclear but it was suggested that the IUD mimics an embryo preventing endometrial cells from releasing PGF2a due to the close contact of the IUD with the endometrium (Rivera Del Alamo *et al.* 2008). In general, mechanotransduction or mechanosensation has been shown to be involved in molecular mechanisms governing epithelial and endothelial cell development and physiology and is mediated via interactions between extracellular matrix, cellular junctions, and the cytoskeleton (Ohashi *et al.* 2017; Sluysmans *et al.* 2017). Moreover, in chondrocytes a mechanism for mechanotransduction has been shown to involve integrin signaling resulting in a reduction of interleukin 1beta (IL1B)-induced nitric oxide and prostaglandin E2 release by decreasing the expression levels of nitric oxide synthase 2 (*NOS2*, iNOS) and prostaglandin-endoperoxide synthase 2 (*PTGS2*, COX-2).

To decipher transcriptomic and proteomic changes in the endometrium, uterine fluid, and conceptus during the time of MRP in the mare a number of “omics” studies have been performed (Hayes *et al.* 2008; Klein *et al.* 2010; Merkl *et al.* 2010; Hayes *et al.* 2012; Klein 2015; Klohonatz *et al.* 2015). Endometrial gene expression changes in response to the presence of a conceptus have been studied between Days 8 and 18 by the use of DNA microarrays and RNA sequencing (RNA-Seq). Changes in the endometrial transcriptome have been detected as early as Day 12 of pregnancy (Merkl *et al.* 2010). Overall, a very complex endometrial response to the presence of the conceptus was observed. The identified gene sets had very heterogeneous biological functions that could reflect i) a response to different embryonic signals and/or ii) differential gene expression in different compartments of the endometrium, such as luminal epithelium (LE), glandular epithelium (GE), and stroma (Klein *et al.* 2010; Merkl *et al.* 2010). For example, many genes differentially expressed between pregnant and cyclic endometrium are known to be regulated by estrogens. Although these endometrial transcriptome studies revealed many new insights into gene expression changes in response to the presence of the equine conceptus, direct conclusions on the equine embryonic pregnancy recognition signal and the mechanisms of MRP in the horse could not be drawn from the obtained results. Thus, these findings call for a deeper analysis of cell-type specific gene expression of the endometrium in response to the equine embryo, which could be the key to understand the mechanism of MRP in the horse. The aim of the present study was to analyze the cell type-specific endometrial response to the presence of a conceptus at the level of gene expression on Day 12 of pregnancy based on the isolation of distinct endometrial compartments by the use of Laser Capture Microdissection (LCM). Day 12 of pregnancy was chosen based on our results from a previous study (Merkl *et al.* 2010) and in order to compare the obtained cell type-specific results to those obtained from the analysis of entire biopsy samples.

Material and Methods

Sample collection and Experimental Design

The sample collection for RNA-Seq of whole biopsy samples has been described previously (Merkl *et al.* 2010). Endometrium biopsies for the isolation of specific endometrial cell types were collected from 6 healthy cycling warmblood mares belonging to the University of Zurich, Clinic of Reproductive Medicine, Switzerland. The mares were monitored for signs of estrus using a LOGIQ e ultrasound equipment (GE Healthcare, Glattbrugg, Switzerland). When a follicle of at least 35 mm diameter was detected in conjunction with an uterine edema, a single dose of 1500 IU human choriogonadotropin (hCG) was applied i.v. to induce ovulation (day -2). The mares were randomly assigned to one of the experimental cycles: control cycle or pregnancy. If assigned to the pregnancy cycle the mare was inseminated with fresh cooled semen one day before ovulation (day -1). For all samples used for the pregnant group, pregnancy was confirmed by ultrasound examination on day 12 after insemination before sample collection when in case of pregnancy the presence of a conceptus can be reliably detected. To induce luteolysis at the end of the experiment, PGF2a was applied on day 12. During the experimental days (-2 to 12) ultrasound images of both uterus and ovaries were recorded and blood samples for measurement of progesterone were collected. Blood samples were centrifuged, blood plasma collected and stored at -20°C until assay. On day 12, endometrial samples were obtained by performing a uterine biopsy. After a longitudinal cut of the biopsy, one half was fixated in formalin for histological examination and the other half was snap-frozen in a cryo-embedding matrix in liquid nitrogen and stored at -80°C.
All the experiments with animals were conducted with the permission of the veterinary inspection office of the Kanton Zurich (permission no. 24/2014) and the degree of severity corresponded to grade 1.

Histological analysis of endometrial biopsies

The endometrial biopsy samples where first evaluated macroscopically and then fixed in a 4% formalin solution for 24 hours. Subsequently, the endometrial samples were embedded in paraffin blocks (Sakura Tissue® Tek® VIP, GMI, Ramsey, MN, USA). The blocks were rinsed with water for 10 minutes, dehydrated in a graded series of alcohols for 5:15 hours (70%, 80%, 96%, 100%), cleared with xylene for 3:30 hours and embedded in paraffin for 3 hours. Then, 2 µm sections were prepared and stained with haematoxylin and eosin according to standard procedure. The histological sections where evaluated and categorized according to Kenney and Doig (Kenney and Doig 1986).

Laser capture microdissection (LCM)

Stained frozen sections (10 µm) were prepared using a microtome cryostat CryoStar NX50 (Histocom AG, Zug, Switzerland) on PEN-Membrane Glass Slides®, and Arcturus® HistoGene® Frozen Section Staining Kit (Applied Biosystems). The collection of luminal epithelium (LE), glandular epithelium (GE) and stromal areas (S) was performed using an ArcturusXT® Laser Capture Microdissection instrument.

RNA isolation, quantification and quality control

RNA isolation was performed with the Arcturus PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific, Darmstadt, Germany). Concentration of the RNA was measured with a *Quantus™* Fluorometer (*Promega*; sensitivity 390 pg RNA/ml using QuantiFluor® RNA Dye). Quality was analyzed by the use of the *Agilent 2100* Bioanalyzer (Eukaryote Total RNA Pico Assay), which estimates the concentration of the RNA sample and provides a quality score (RNA integrity number, RIN).

Illumina RNA-Sequencing and data analysis

For RNA derived from whole biopsy samples, the mRNA-Seq sample preparation kit (Illumina, San Diego, USA) was used for preparation of RNA-Seq libraries as described recently (Samborski *et al.* 2013). For each group (Day 12 pregnant and non-pregnant cyclic mares), 4 RNA-Seq libraries were prepared corresponding to samples from 4 mares, respectively. Sequencing of the libraries was conducted on an Illumina Genome Analyzer IIx system. Single-end reads (76 bp) were generated using Cluster generation single read, cBot kit and Cycle sequencing kit v4 (Illumina). Each RNA-Seq library was analyzed on one lane of a single-read flow cell (in total 8 lanes).
For the generation of RNA-Seq libraries from LCM samples, the Ovation® Single Cell RNA-Seq System (NuGEN Technologies, Inc., European office, Leek, The Netherlands) was used starting from 1 ng total RNA. Since for a number of libraries the concentration of the obtained cDNA fragments was too low (Agilent Bioanalyzer DNA High Sensitivity assay), an additional amplification was performed with the KAPA HiFi Library Amplification Kit (Axon Lab AG, Baden, Switzerland). Single-end reads (125 bp) were generated on an Illumina HiSeq 2500 instrument running multiplexed libraries on two lanes of one flow cell.
The obtained sequence reads (Fastq files) of both studies were analyzed with the same data analysis pipeline on a locally installed version of Galaxy (Giardine *et al.* 2005) essentially as described recently (Kradolfer *et al.* 2016). Potential PCR duplicates were removed using the tool FastUniq (Galaxy Version 1.1). The most current GFF3 annotation file from NCBI was used for mapping and assignment of mapped reads to genes. Library sizes (reads mapped to annotated genes) were on average 598,000 (ranging from 138,000 to 1,467,000). All analysis steps after generation of the read count table were performed using the BioConductor package EdgeR (Robinson *et al.* 2010) according to the EdgeR users guide including the identification of differentially expressed genes (DEGs). Due to the low number of biological replicates in some LCM sample groups and the suboptimal RNA quality, a rather explorative approach was used to identify DEGs and to reduce the number of false positives. This consisted of two approaches; i) filtering of DEGs based on read counts/counts per million (CPM) of individual samples, and ii) checking expression in LCM samples of genes differentially expressed (DE) in the complete biopsy samples to assign spatial gene expression information. The filtering was performed by checking the CPM of each DEG revealed by the EdgeR analysis if similar expression was detected in each sample of the group with higher expression and expression was lower in all samples of the group with on average lower expression. For example, if read counts for C_S_B, C_S_M, P_S_M, P_S_U (C, P: control, pregnant; S: stroma; last letter: code for the mare) were 1, 0, 534, 3, this

gene was not considered as DEG. Also, if read counts were 128, 2, 156, 98, such genes were removed from the DEG list. Also, for LCM sample groups with more replicates, a similar filtering was used. In the second approach, the probable location of gene expression of DEGs found in the whole biopsy was indicated by the expression data from the LCM samples, i.e., if CPM were very low or 0 in two cell types and higher in the third cell type. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE112236 (LCM samples) and GSE112237 (biopsy samples).
Venn diagrams were generated with the web tool jvenn (Bardou *et al.* 2014). Integrated analysis of different functional databases was done using the “Functional annotation clustering” and “Functional Annotation Charts” tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8) (Dennis *et al.* 2003). Graphical illustration of overrepresented gene ontologies and other functional categories was produced with the Cytoscape v3.0.0 application ClueGO v2.0.3 (Bindea *et al.* 2009). Hierarchical clustering of DEGs was performed with the HCL tool of Multi Experiment Viewer (MeV) v4.8.1 (Saeed *et al.* 2003) based on mean-centered normalized log2 transformed read counts (normalized value of a sample minus average of all normalized values).

Results

Histological analysis of endometrial biopsies and serum progesterone concentrations

Signs of inflammation and grade of endometrial fibrosis are the markers used to estimate the mare's ability to conceive and maintain a pregnancy. The endometrium biopsies collected for LCM were graded between I (normal endometrium) and II A (moderate inflammation and multifocal-diffuse fibrosis).

During estrus, serum progesterone (P4) concentrations in serum were always below 1 ng/ml (Supplemental Table 1). Except for two values, the P4 concentration on the day before ovulation were still below 1 ng/ml. During diestrus, concentrations were between 5 and 20 ng/ml with high individual differences.

RNA isolation from cells isolated by Laser Capture Microdissection and RNA sequencing

Cells from luminal epithelium (LE), glandular epithelium (GE) and stromal (S) areas were collected by Laser Capture Microdissection (LCM) from stained frozen sections prepared from endometrial biopsies collected on Day 12 of pregnancy and Day 12 of the estrous cycle, respectively. The RNA yield from the cells isolated by LCM was between 0.5 and 18 ng. The samples used for RNA-Seq showed partial degradation but were at least in a similar quality range (RIN 5-7). RNA-Seq libraries were produced using samples from 5 mares, i.e., a non-pregnant control cycle and a pregnancy cycle of each mare corresponding to 30 RNA samples. For most of the 30 samples cDNA fragments could be amplified in sufficient amounts for sequencing. Twenty-one out of 30 libraries were selected for sequencing based on the results of the library product analysis. Altogether, data derived from 19 samples was found to be useful for statistical analysis of gene expression (LE pregnant: n=3, LE cyclic: n=5, GE pregnant: n=4, GE cyclic: n=3, stroma pregnant: n=2, stroma cyclic: n=2). A pairwise distance heatmap illustrating the correlation of the expression profiles of the samples (Figure 1A) and a principal component analysis (Figure 1B) indicated that gene expression differences between LE, GE and stroma were greater than differences between pregnant and cyclic samples of the respective cell types. Since the distance of LE samples to the stroma and GE samples were greatest for principal component 1 the gene expression in LE was found as most distinct compared to the other cell types. The most significant differences between pregnant and cyclic mares were observed for LE samples.

Comparison of RNA-Seq of whole biopsy samples and cell type-specific samples collected by LCM

The number of detectable genes (reaching a threshold of read counts) was compared between the data sets for whole biopsies and LCM samples (Figure 2). In the RNA-Seq analysis of the biopsies, 3806 genes were detected which were not detectable in the LCM samples. Altogether, 175 genes were detectable in LE, GE, and/or stroma but not in the analysis of the biopsy samples (Figure 2). Interestingly, the highest number of specifically expressed genes in the LCM data sets was obtained for LE. The lowest number of detectable genes (6880) was observed for stroma. A relatively high number of genes was detected in both epithelia but not in the stromal area (2725 genes).

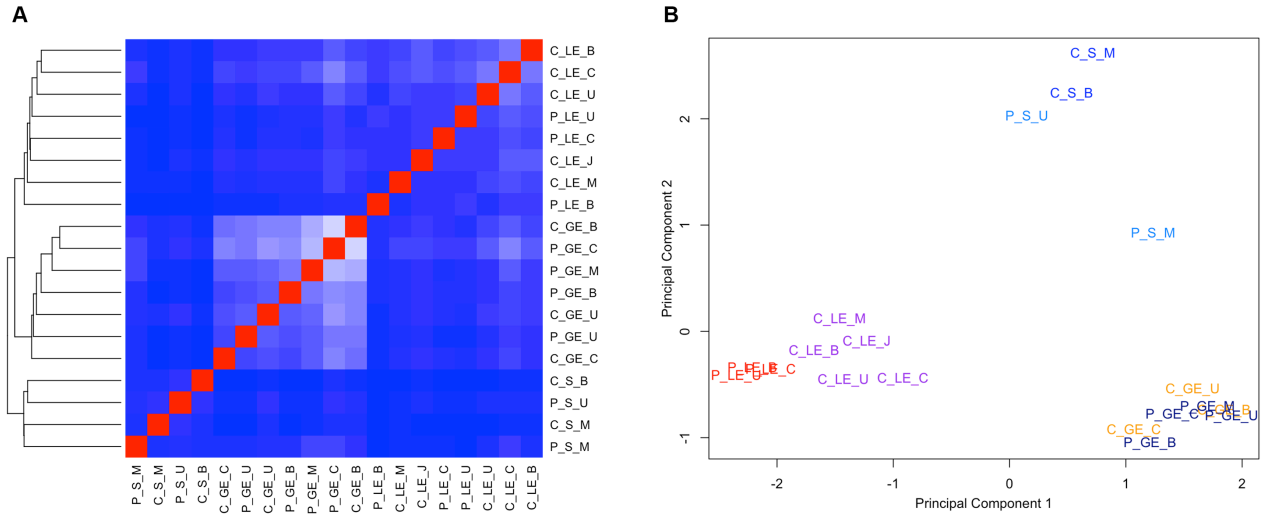

Figure 1: Pairwise distance heatmap (Geneplotter) (A) and principal component analysis (Multidimensional Scaling Plot, EdgeR) (B) for LCM data sets. P: pregnant, C: cyclic control, LE: luminal epithelium, GE: glandular epithelium, S: stromal areas, last letter: initial of the mare's name.

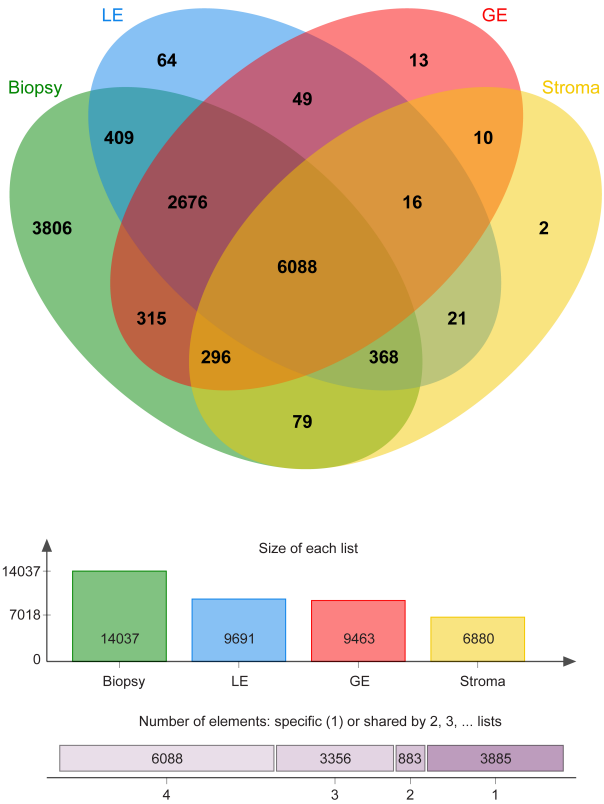

Figure 2: Venn diagram for comparison of detectable genes in the RNA-Seq analyses of endometrial biopsies and cell type-specific samples isolated by Laser Capture Microdissection. LE: luminal epithelium, GE: glandular epithelium.

Analysis of differential gene expression

Because of the effects of the low RNA concentration and the partial degradation of the RNA samples on the quality of the RNA-Seq data (increased variation between individual samples), a rather explorative strategy for identification of DEGs had to be applied. The first approach was based on a statistical analysis of the RNA-Seq data for the LCM samples and a rigorous filtering of the obtained DEGs to minimize false positive results. Furthermore, a comparison between the cell type-specific data and the available RNA-Seq data set for endometrium biopsy samples from day 12 of pregnancy and cycle was performed (Bauersachs and Wolf 2012; Bauersachs and Wolf 2015). Here, the results from the cell type-specific data were used to identify DEGs obtained from the analysis of complete endometrium biopsies of cyclic and pregnant mares which are specifically expressed in the endometrial compartments. The numbers of identified DEGs for endometrial biopsy analysis and for the analysis of specific endometrial cells are shown in Table 1. The Venn diagram in Figure 3 shows the overlap of DEGs between whole biopsy, LE, GE and stroma. For 135 out of the 502 DEGs obtained from the analysis of biopsy samples, the localization of differential gene expression could be determined. Furthermore, 185 DEGs were only found in the analysis of samples isolated by LCM, most of them showing specific expression in LE or stroma. The number of obtained DEGs in GE was low (42 genes), whereas the analysis of LE (165 genes) und stroma (154 genes) revealed a higher number of DEGs. A hierarchical cluster analysis of the relative gene expression (mean-centered) across all LCM samples for the DEGs identified by the first approach (statistical analysis of LCM results and DEGs filtering) showed that many of the DEGs are specifically expressed or downregulated in the respective cell type (Supplemental Figures 1-3). A more detailed view of the DEGs for the LCM samples is shown in the Venn diagram in Figure 4. Whereas the number of genes with higher (upregulated) or lower expression (downregulated) in samples collected from pregnant mares was similar for LE and GE, the majority of DEGs in stroma showed higher expression for pregnant mares (upregulated). Also for the analysis of biopsy samples more upregulated than downregulated genes were found. In addition, the comparison of LE and stroma revealed some genes with opposite regulation, i.e. higher expression in stroma of pregnant mares but lower expression in LE compared to non-pregnant cyclic controls.

Table 1: Overview of obtained differentially expressed genes for day 12 of pregnancy in comparison to day 12 of the estrous cycle

Sample	Higher in pregnancy	Lower in pregnancy	Tot al
Biopsy	234	139	373
Luminal epithelium	94	71	165
Glandular epithelium	17	25	42
Stroma	138	16	154

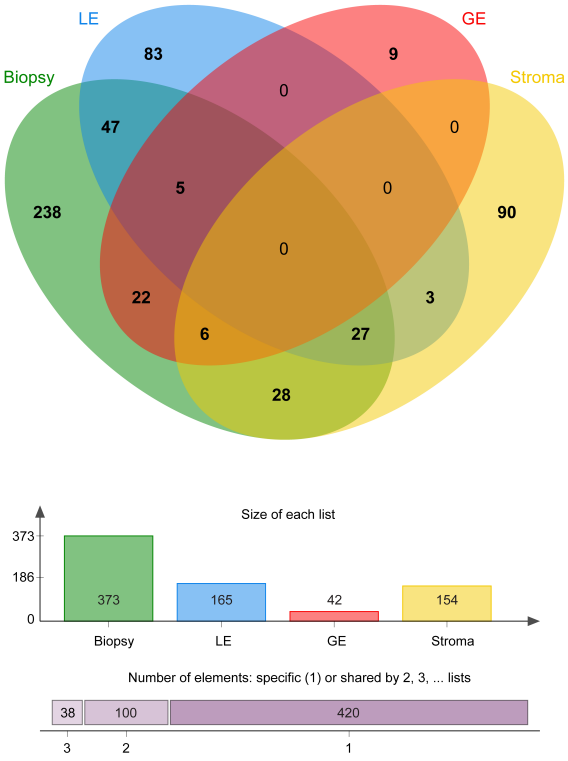

Figure 3: Venn diagram for comparison of differentially expressed genes in different endometrial compartments (LCM samples) and complete endometrial biopsy. LE: luminal epithelium, GE: glandular epithelium.

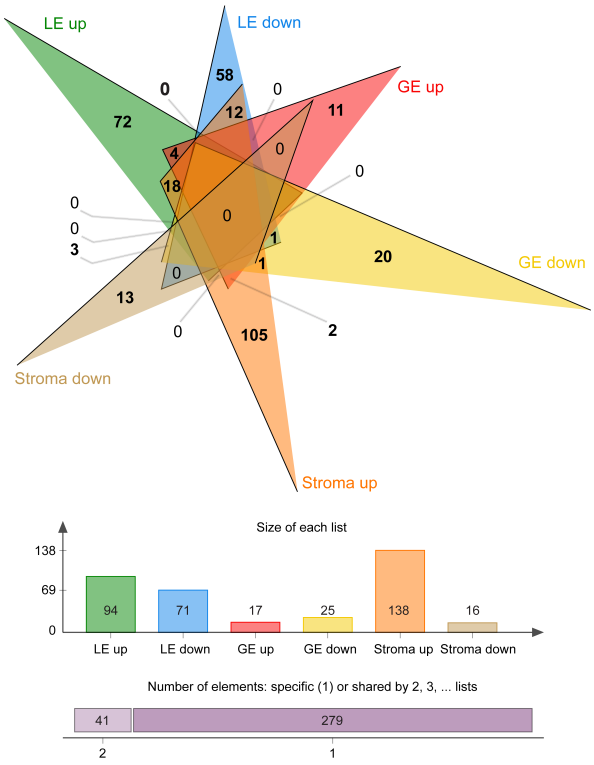

Figure 4: Venn diagram for comparison of differentially expressed genes in different endometrial compartments (LCM samples) in consideration of up- and downregulation in Day 12 pregnant vs. cyclic endometrium. LE: luminal epithelium, GE: glandular epithelium.

Overrepresentation analysis of differentially expressed genes

A functional classification of the obtained DEGs for the single endometrium compartments and for the complete biopsy data set was performed to identify overrepresented functional categories. DAVID Functional Annotation Clustering (Dennis *et al.* 2003) was performed for genes up-regulated or down-regulated in endometria from day 12 pregnant mares compared to day 12 cyclic controls (Supplemental Table 3). For all cell types and complete biopsy samples, less significant annotation clusters were obtained for down-regulated genes. Genes up-regulated in LE were enriched for processes/functions associated with secretion, development, signaling, immune response, and proteolysis and cellular components related to vesicles and membrane. Overrepresentation of down-regulated genes in LE was only found for a few groups of functional categories with relatively low numbers of assigned genes. These categories were related to retinoid metabolism, cell motility/migration, and signaling. Due to the low number of DEGs for GE, no significant annotation clusters were obtained for GE and Functional Annotation Chart analysis was done instead. Functional terms enriched for genes up-regulated in GE represented secretory processes, reproductive processes, and signaling. Some categories involved in secretory processes were also enriched for genes down-regulated in GE. The majority of the other enriched functional categories was related to various metabolic and catabolic processes. For stroma, genes identified as up-regulated in samples from pregnant mares were overrepresented for a variety of processes/functions, such as wound healing, secretion, cell migration, development, signaling and cell communication, metabolism and biosynthesis, angiogenesis, cell adhesion, and gene expression. Enriched cellular components were related to vesicles and membrane, focal adhesion and cell junction, and contractile fiber. Genes down-regulated in stromal areas of endometria from pregnant mares were enriched for processes involved in lipid and DNA metabolism, cytoskeleton, and reproduction. In the complete biopsy, most of the overrepresented functional categories were also obtained for the LCM samples. Some processes/functions were not found as overrepresented for the LCM samples, e.g., a number of immune function processes, unsaturated fatty acid and icosanoid metabolic process, ion channel, and terms related to blood vessel development. Genes down-regulated in complete biopsies were mainly enriched for metabolic processes and extracellular matrix.

To perform a comparison of the functional annotation results, all functional terms to which genes up-regulated in pregnant mares could be assigned were analyzed between the different cell type data sets and the biopsy data set (Supplemental Table 4). Most of the functional terms related to blood vessel development and angiogenesis were significantly enriched only for the biopsy sample. Many terms containing genes involved in immune response and response to various factors were enriched for the complete endometrial biopsy. A number of those terms was also enriched in LE and/or stroma. The same was found for terms related to proliferation, cell death, and metabolic processes. Of those, some specific terms were only enriched for LE, such as terms related to protein glycosylation. Cell adhesion and junction terms were mainly found as overrepresented for the complete biopsy. Terms for extracellular matrix and connective tissue development were enriched for biopsy and LE. Secretion and extracellular vesicle were found as enriched for biopsy and LCM samples. Overall, the comparison revealed the localization of differential gene expression for overrepresented functional terms.

Furthermore, some overrepresented functional terms were found which were not significant for the complete biopsy sample but for a specific cell type.

Finally, DEGs were compared to a number of gene lists assigned to pathways involved in prostaglandin synthesis and regulation, steroid hormone biosynthesis, and estrogen, oxytocin, prolactin, interferon and IL-1 signaling (Supplemental Table 5). Most of the assigned genes were found as upregulated, but mainly only in the analysis of complete biopsy samples. For the genes found as differentially expressed in the LE 7 were downregulated and 5 upregulated, genes in GE and stroma were mostly upregulated. The genes assigned to PG metabolism and signaling were involved in the release of PG precursor molecules (*PLA2G2A* down in LE, *PLA2G4F* up in biopsy) from the plasma membrane, PG transport (*ABCC1* up in LE and GE, *SLCO2A1* up in biopsy), or function as PG receptors (*PTGER4* up in biopsy, *PTGFR* down in biopsy). In addition, two annexin genes were found, *ANXA1* downregulated in stroma and *ANXA2* upregulated in LE. In agreement with our previous findings (Merkl *et al.* 2010), Oxytocin receptor (*OXTR)* mRNA was upregulated (stroma) and estrogen receptor alpha (*ESR1*) mRNA downregulated in the complete biopsy, but upregulated in stroma.

Discussion

The motivation to study gene expression in individual endometrial cell types was based on previous findings in the mare and other species indicating distinct differences in gene expression between endometrial compartments in response to embryonic signals and during the estrous cycle (Niklaus and Pollard 2006; Merkl *et al.* 2010; Bauersachs and Wolf 2012; Filant and Spencer 2013; Field *et al.* 2015; Brooks *et al.* 2016; Rosario *et al.* 2016). Furthermore, the endometrial epithelia, particularly the LE, are thought to play important biological roles in conceptus survival, growth, and implantation in mammals showing non-invasive placentation (Spencer *et al.* 2007; Brooks *et al.* 2016; Spencer *et al.* 2016). Since the percentage of LE in an endometrial biopsy sample is relatively low, many specific changes in gene expression probably remain undetectable when analyzing whole endometria due to dilution and averaging effects. Furthermore, with respect to data analysis and interpretation, it is difficult to draw conclusions on involved pathways if the localization of gene expression regulation is unknown.

The present study revealed that LE, GE and stroma of the equine endometrium have specific transcriptome profiles and respond specifically to conceptus signals which is in agreement with findings in other species (Niklaus and Pollard 2006; Field *et al.* 2015; Brooks *et al.* 2016). Interestingly, with respect to differential gene expression the difference between LE and GE seems to be greater than between GE and stroma. The main response to the presence of a conceptus, i.e. highest number of DEGs was found in the LE. In contrast, more DEGs were found in GE than in LE in the course of the preimplantation phase in a recent study in sheep (Brooks *et al.* 2016). Although in a number of studies, transcriptome analysis of endometrial LCM samples has been performed, so far, no study compared the three main compartments and complete endometrial samples.

As expected, a higher number of detectable genes was present in the data set for complete endometrial biopsies. Since the preparation of RNA-seq libraries started from only 500 pg of total RNA (corresponding to 50 cells or less) and the quality of the RNA from LCM samples was lower compared to the biopsy samples, some transcripts present in very low copy numbers probably got lost during library preparation. Nevertheless, expression of some genes was only detectable in the LCM samples, which could be due to specific expression in certain cell types, making detection in a whole endometrium biopsy very difficult because of the dilution effect. In addition, the LCM samples for stroma were cut from regions without visible blood vessels, why the biopsy samples may contain transcripts specific for endothelial cells and blood vessel associated immune cells which are not present in the LCM samples.

Despite the limitation due to the suboptimal quality of the RNA isolated from the LCM samples and the low number of replicates, the obtained data could be used to identify cell type-specific differential gene expression by applying stringent filtering criteria. This revealed localization of differential gene expression in the endometrium and identification of DEGs not detectable in complete biopsy samples because of averaging and dilution effects. Even opposite regulation of gene expression was found for a number of genes in LE and stroma. So far, this has not been reported. In a few studies, cell type-specific endometrial expression of transcripts or proteins has been studied during the preimplantation phase for selected targets (Spencer *et al.* 2008; Ka *et al.* 2009; Silva *et al.* 2011; Seo *et al.* 2014a).

The data analysis of the current study revealed enrichment of specific functional categories and biological processes for the investigated cell types, thereby localizing regulatory processes in response to the conceptus to functional compartments of the endometrium. In the following, the results obtained for functional groups of genes potentially important for conceptus-endometrial interactions are discussed. However, although this study contains a lot of interesting new information, due to limitations in the number of biological replicates and the quality of RNA isolated from LCM samples, it is rather to see as proof-of-concept study with an explorative character. The reader should be aware that before starting more in-depth functional studies on interesting genes identified in this study, these findings should be first validated.

Genes related to angiogenesis and blood vessel development.

In our previous study, one predominant complex of overrepresented categories was related to angiogenesis and blood vessel development (Merkl *et al.* 2010). The analysis of the DEGs for the complete biopsy sample confirmed this finding. The DEGs for the individual cell types did not show highly significant enrichment for these terms, except for LE where 'vasculature development' was significantly enriched with 11 genes. In contrast to the DEGs assigned to 'vasculature development' identified in the complete biopsy samples, the DEGs in LE were not classical angiogenesis or vessel development genes. Based on their known functions, most of those genes have a more indirect role in stimulation of vascular development and differentiation suggesting a paracrine effect on blood vessels. For example, angiopoietin like 4 (ANGPTL4), fibroblast growth factor 9 (FGF9), C-X-C motif chemokine ligand 17 (CXCL17), nephroblastoma overexpressed (NOV), and Wnt family member 7B (WNT7B) could be factors expressed in LE and having effects on vasculature development. In the mouse, expression of Angptl4 mRNA has been shown to increase in implantation segments during decidualization and in endometrial stromal cells undergoing decidualization in vitro (Scott *et al.* 2012). ANGPTL4 has been shown to function in modulation of vascular permeability, angiogenesis, and inflammatory signaling (Guo *et al.* 2014) but also in regulation of triglyceride metabolism (Mattijssen and Kersten 2012). FGF9 is affecting blood vessel development and functions indirectly via induction of smooth muscle cell proliferation and wrapping of microvessels (Agrotis *et al.* 2004; Frontini *et al.* 2011). CXCL17 has been shown to induce the production of proangiogenic factors such as vascular endothelial growth factor A (VEGFA) from treated monocytes (Lee *et al.* 2013). Increased expression of galectin 3 binding protein (*LGALS3BP*) mRNA was detected in LE similar to findings in cattle (Bauersachs *et al.* 2006). Expression of this gene has been described in association with immunomodulatory effects (Laubli *et al.* 2014). Furthermore, *LGALS3BP* expression in tumor cells has been shown to induce *VEGF* expression and to stimulate blood vessel formation (Piccolo *et al.* 2013). NOV has been shown to directly stimulate endothelial cells and to induce angiogenesis in vivo (Lin *et al.* 2003). WNT7B could also have an effect on blood vessel formation and remodeling in the endometrium based on findings in other tissues, e.g., in renal medullary capillary development (Roker *et al.* 2017), and furthermore, the proposed role of the WNT signaling pathway in placenta formation (Cross *et al.* 2003). The DEGs identified in the complete biopsy samples and assigned to 'vasculature development' contained a number of typical genes involved in angiogenesis and blood vessel remodeling and formation, such as members of the vascular endothelial growth factor pathway,

angiopoietin system, structural molecules of endothelial cells, and angiogenesis-related transcription factors. Since the samples isolated by LCM did not contain blood vessels (stromal areas containing visible vessels were not selected for cutting), the differential expression of these genes is most likely located in endothelial cells. Furthermore, the increase of expression of endothelial markers suggests an increase of vascularization in response to the presence of the conceptus. In agreement with this idea are previous findings that revealed a role of the conceptus in directing vasculogenesis and endometrial vascular perfusion during early pregnancy (Silva *et al.* 2005; Silva *et al.* 2011).

Genes involved in immune response.

Another group of highly enriched functional categories for genes upregulated in complete endometria samples was related to immune response. “Inflammatory response” was significantly enriched for complete biopsy but also found as overrepresented for LE. The corresponding genes upregulated in LE were mainly involved in negative regulation of inflammatory response. The products of *C5orf30* (Muthana *et al.* 2015), *CXCL17* (Oka *et al.* 2017), *NOV* (Liu *et al.* 2014), *TNFRSF21* (Liu *et al.* 2001), and the complement inhibitors CD55 (Palomino *et al.* 2013), SERPING1 (Beinrohr *et al.* 2011) have been shown to modulate and suppress immune response. In the context of induction of immune tolerance, *CD200* (Clark *et al.* 2015) has been described as tolerance-signaling molecule in humans and was found as expressed in LE and stroma and upregulated in the complete biopsy in the present study. In contrast, the genes upregulated in the complete biopsy were predominantly known as associated with activation of immune response such as complement components, activating chemokines, and genes expressed in activated cells of the specific immune system. Interestingly, upregulation of the mRNA for TNF receptor superfamily member 18 (*TNFRSF18*) was found in complete biopsy samples with very low but increased expression in samples from pregnant mares. The gene *TNFRSF18* is known to be expressed at high levels in activated T cells and regulatory T cells (Ronchetti *et al.* 2015). Overall, based on the genes found as upregulated in response to the presence of an embryo pregnancy, the immune system is modulated in a way to inhibit inflammatory response and induce tolerance.

Genes with probable functions in interaction with the conceptus.

Genes involved in positive regulation of cell proliferation and negative regulation of apoptosis were overrepresented for LE (upregulated genes). Many of the genes involved in cell proliferation are known to function as autocrine or paracrine growth factors or growth factor receptors. The genes *FGF9, WNT7B*, and *NOV* were already discussed above in context of their potential role on blood vessel development. In addition to its effects on angiogenesis, FGF9 has been identified as an autocrine E2-stimulated endometrial stromal growth factor (Tsai *et al.* 2002). In the pig, FGF9 protein was localized in the glandular epithelium on Day 14 of pregnancy and suggested to function as an embryonic growth factor (Østrup *et al.* 2010). In equine endometrium, expression of *FGF9* was mainly found in LE derived from Day 12 pregnant mares. The gene *FGFR2* encoding a high-affinity FGF9 receptor, was expressed in LE and GE. It is very likely that *FGF9* expression in the mare is also induced by E2 from the conceptus like in the pig and by ovarian E2 in humans, but has a distinct localization and has

autocrine effects on the LE and/or paracrine effects on the GE as well as on the conceptus that has been shown to express *FGFR1-4* mRNAs (de Ruijter-Villani *et al.* 2013). Insulin like growth factor binding protein 3 (*IGFBP3*) mRNA expression has been shown to be upregulated by P4 in LE of ovine endometrium (Satterfield *et al.* 2008). Secretion of IGFBP3 by human endometrial stromal cells was found to be stimulated in co-culture with mouse embryos suggesting a regulation by embryonic signals (Liu *et al.* 1995). In our study, *IGBFP3* was mainly expressed in LE and a significant upregulation was found for the whole biopsy samples. Indian hedgehog (*IHH*) expression is important for uterine P4-mediated paracrine signaling in context of stromal decidualization and embryo implantation (Wetendorf and DeMayo 2012). Interestingly, in equine endometrium, *IHH* expression was found in all three cell types, but in LE only in samples derived from pregnant endometrium. Expression of stanniocalcin (*STC1*) mRNA, encoding a protein involved in regulation of intracellular calcium and phosphate (Song *et al.* 2006), was found in all three cell types but was strongest in LE. In the pig, *STC1* has been described as a marker for implantation exclusively expressed in the luminal epithelium (Song *et al.* 2009) whereas in sheep expression was predominantly detected in the endometrial glands after Day 16 of pregnancy (Song *et al.* 2006). In the mouse uterus, *STC1* gene expression is confined to the LE in the non-pregnant state and shifts to mesometrial stromal cells bordering the uterine lumen after implantation suggesting a role in the process of decidualization (Stasko *et al.* 2001). Based on its specific localization of expression at the embryo-maternal interface, the secreted STC1 protein could have effects on the conceptus as well as on other endometrial cells. The distinct differences in *STC1* gene expression compared to other species could indicate a specific function in regulation of conceptus attachment and implantation, which occurs much later in the mare.
With respect to the very long preimplantation phase in the horse, cell adhesion-related gene expression in the LE is particularly interesting. In agreement with a recent study on mucin 1 (MUC1) expression (Wilsher *et al.* 2013), *MUC1* and *MUC3* mRNA was mainly detected in LE and increased in samples from Day 12 pregnant mares.

Genes related to prostaglandin metabolism and signalling.

Annexin proteins are involved in the regulation of prostaglandin production. In our study annexin A1 (*ANXA1*) was downregulated in stroma and *ANXA2* upregulated in LE. In a recent study, annexin proteins (*ANXA1*, *ANXA2*, *ANXA5*) were found in uterine flushing on day 14-16 of pregnancy in sheep (Romero *et al.* 2017). Annexin A2 protein has been found as increased in porcine endometrium on Day 12 of gestation compared to Day 12 of the estrous cycle (Moza Jalali *et al.* 2016). Furthermore, ANXA2 has been shown to be involved in embryo attachment to the endometrium in the mouse and in humans (Garrido-Gomez *et al.* 2012; Wang *et al.* 2015). Expression of *ANXA2* mRNA was detected in all three cell types in equine endometrium but specifically increased in LE. Since ANXA2 is a multifunctional protein, its role during early pregnancy in the mare is unclear. It can be speculated that it is involved in the regulation of PG synthesis. The finding in human endometrium that ANXA2 and RhoA are co-localized with the F-actin network in epithelial cells (Garrido-Gomez *et al.* 2012) and the role of Rho kinases and actin cytoskeleton in mechanosensation, could be a link of sensing mechanical stimuli from the migrating conceptus to the regulation of PG synthesis.

In a recent study on endometrial phospholipase A2 isoform expression in equine endometrium, *PLA2G4A* and *PLA2G2A* expression was negatively correlated with progesterone (P4) concentrations (Ababneh and Troedsson 2013). Endometrial PLA2 gene expression is probably involved in controlling PGF2a production by releasing PG precursor molecules from the plasma membrane. In our study we found *PLA2G2A* downregulated in LE and *PLA2G4F* upregulated in the complete biopsy, suggesting a distinct local regulation of PLA2 gene expression. In addition to the release of PG precursors from the membrane, PG transport plays an important role in PG regulation. The mRNAs for the prostaglandin transporter genes *ABCC1* and *SLCO2A1* have been shown to be expressed in porcine endometrium during the estrous cycle and early pregnancy (Seo *et al.* 2014b; Jang *et al.* 2017). The expression of *ABCC1* in porcine endometrium was mainly localized in LE and GE (Jang *et al.* 2017), whereas *SLCO2A1* expression was found in LE and blood vessels (Seo *et al.* 2014b). In equine endometrium, upregulation of *SLCO2A1* expression was so far only found on day 22 of pregnancy (Atli *et al.* 2010). In our study, *ABCC1* expression was significantly increased in the whole biopsy samples. The data from LCM samples revealed expression in LE and GE with higher values in samples from day 12 of pregnancy similar to the expression pattern in the pig. The transcript for *SLCO2A1* was also significantly increased in the whole biopsy samples, but not detectable in the LCM samples supporting that endometrial *SLCO2A1* expression is mainly located in blood vessels. Regulation of expression of classical genes involved in PGE2 and PGF2a synthesis, such as *PTGS2* (also known as *COX2*) and PG synthases was not found on Day 12 of pregnancy. Only *PTGIS* was upregulated in the whole biopsy samples. In equine endometrium, PTGIS protein expression was detected in LE and GE during mid-luteal phase (Rebordão *et al.* 2017). In the LCM samples, *PTGIS* mRNA expression was also found in LE and GE with higher values in samples collected from Day 12 pregnant mares (not significant). However, the specific functional of *PTGIS* in the endometrium is not known.

The mRNA for the hyaluronic acid receptor CD44 was downregulated in LE LCM samples and upregulated in stroma and complete biopsy of Day 12 pregnant mares. CD44 is involved in a variety of processes. With respect to PG metabolism, CD44 signalling has been found to activate PTGS2 and generate VEGF resulting in increased proliferation of endothelial cells (Murphy *et al.* 2005). The results found in this previous study are in agreement with the localization of *CD44* expression observed in our study. Since regulation of *PTGS2* at the level of transcription was not observed, regulation at the protein level could be possible. A closer look at the RNA-seq data revealed that *PTGS2* expression is low in whole biopsy samples and in LCM samples only detectable in LE. The expression of other PG synthases such as *PGFS* and *PGES3* was also detected, but in all endometrial compartments.

Similar to findings for Days 14 and 21 (de Ruijter-Villani *et al.* 2015), PGF2a receptor mRNA (*PTGFR*) was downregulated in the whole biopsy samples, but not detectable in LE, GE, and stroma suggesting main expression in blood vessels. PGF2α) secreted from the endometrium is a major luteolysin. Furthermore, there is evidence that the mare has like some other mammals an PGF2a auto-amplification system in which PGF2a can stimulate its own production and *PTGFR* downregulation is uncoupling this system and thereby contributing to prevention of luteolysis (de Ruijter-Villani *et al.* 2015; Kozai *et al.* 2016).

In agreement with our recent study, the mRNA for oxytocin receptor (*OXTR*) was upregulated in whole biopsy samples. The data from LCM samples revealed expression in all compartments and upregulation in GE (medium confidence). Estrogen receptor 1 mRNA (*ESR1*) was slightly downregulated for the whole biopsy dataset (FDR 0.034) confirming our and other recent findings (Klein *et al.* 2010; Merkl *et al.* 2010). However, the LCM data revealed expression in LE, GE, and stroma with increased expression in stroma collected from Day 12 pregnant mares. Due to the relatively high variation of expression between biological replicates, this has to be further confirmed. Not only for the mare, *OXTR* and *ESR1* have been suggested to play a major role in the oxytocin-PGF2a feedback loop involved in regulation of luteolysis (de Ruijter-Villani *et al.* 2015).

In conclusion, this study revealed new and important spatial information about endometrial gene expression during the phase of initial recognition of pregnancy in the mare using laser capture microdissection to specifically study LE, GE, and stromal compartments. The results indicate a direct and/or indirect (via LE) induction of angiogenesis and blood vessel development by the presence of the conceptus. Expression changes of immune-related genes suggest a specific spatial regulation of the immune system. Differential expression of many growth factors involved in embryo-maternal interaction and intraendometrial communication controlling cell differentiation and endometrial remodeling were identified. Finally, expression of genes involved in PG metabolism and signaling indicates transcriptional regulation of genes functioning in production of PG precursor, PG transport, and PG receptors, specifically PTGFR in context of prevention of luteolysis.

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Conflicts of Interest

The authors declare no conflicts of interest.

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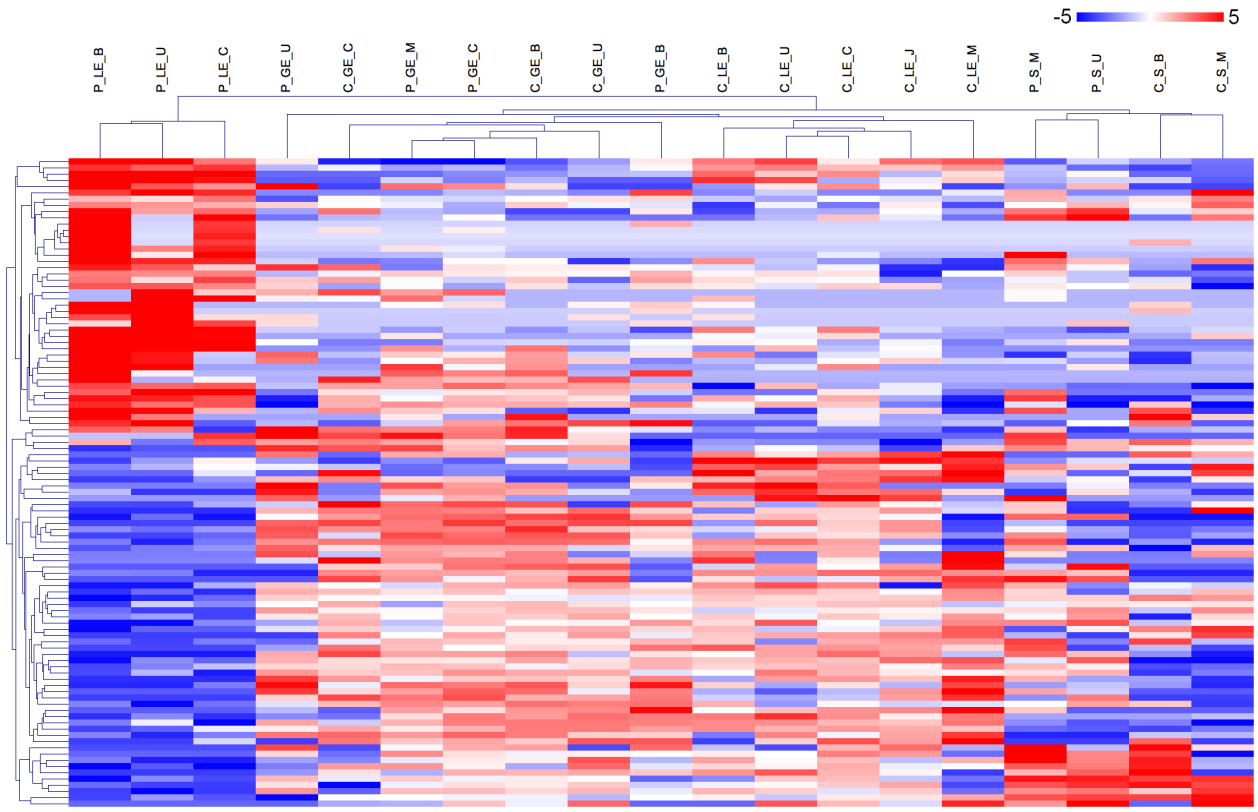
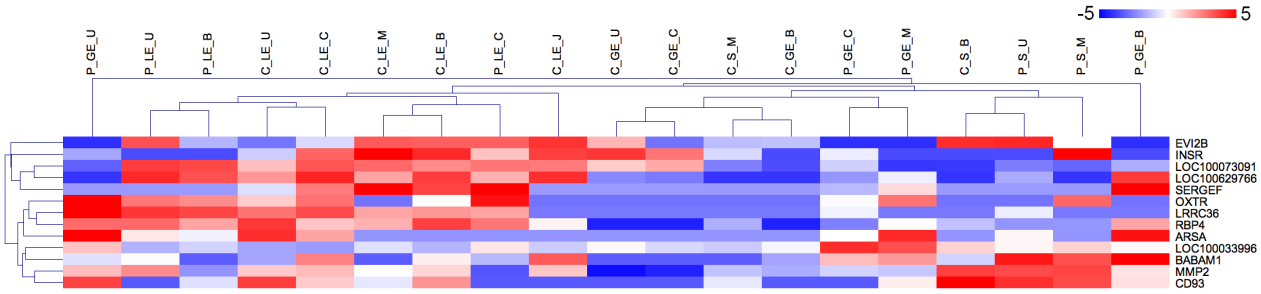
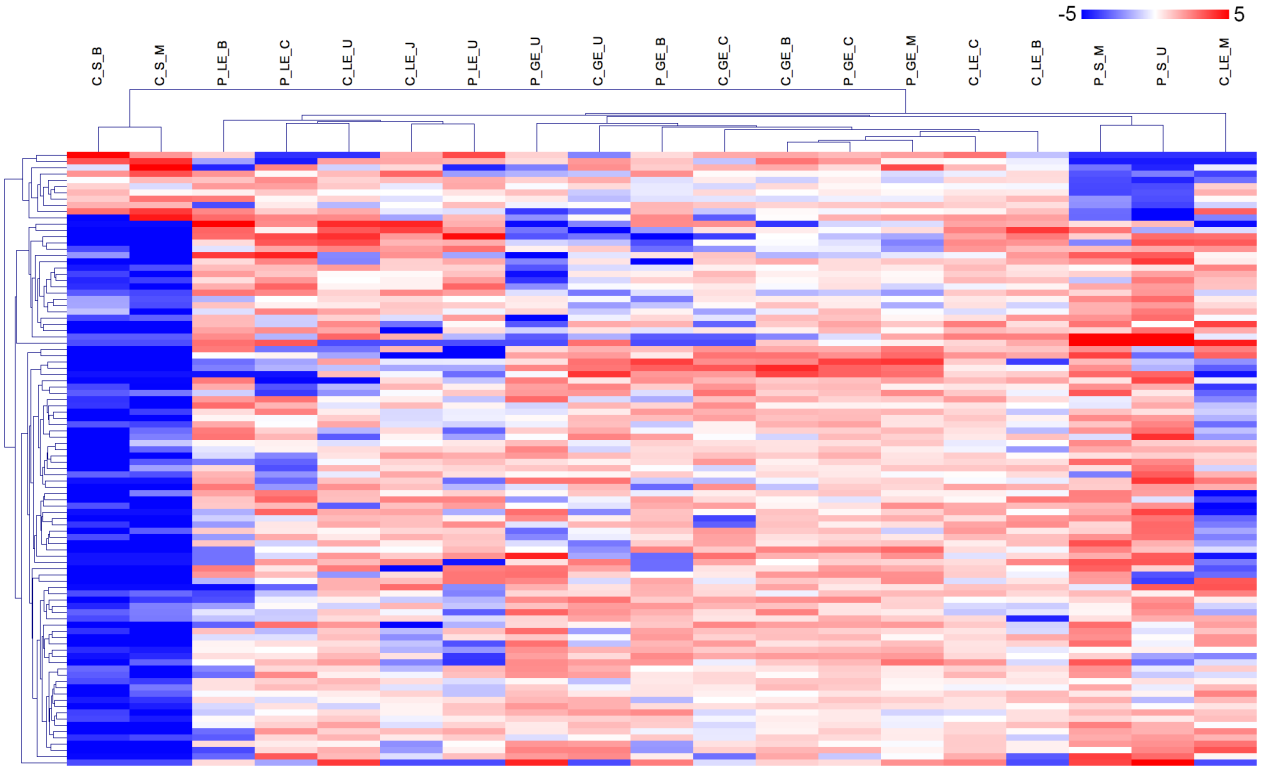
Anhänge



Supplemental Figure 1: Hierarchical cluster analysis of differentialy expressed genes in luminal epithelium (LE). Mean-centered expression values (log_2 scale) were calculated and used for hierarchical cluster analysis (MeV_4_8 v10.2). GE: glandular epithelium, S: stromal areas, P: pregnant, C: cyclic control, last letter of sample ID: initial of mare's name.



Supplemental Figure 2: Hierarchical cluster analysis of differentialy expressed genes in glandular epithelium (GE). Mean-centered expression values (log_2 scale) were calculated and used for hierarchical cluster analysis (MeV_4_8 v10.2). LE: luminal epithelium, S: stromal areas, P: pregnant, C: cyclic control, last letter of sample ID: initial of mare's name.



Supplemental Figure 3: Hierarchical cluster analysis of differentialy expressed genes in stroma. Mean-centered expression values (log_2 scale) were calculated and used for hierarchical cluster analysis (MeV_4_8 v10.2). LE: luminal epithelium, GE: glandular epithelium, S: stromal areas, P: pregnant, C: cyclic control, last letter of sample ID: initial of mare's name.

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